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Breast Cancer Susceptibility Gene

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<b>13. ABSTRACT (Maximum 200 Words)</b>  Besides family history of cancer and an individual's age, no single etiologic factor can identify women at an increased risk for the disease. Approximately 10% of all cases of breast cancer exhibit a familial pattern of incidence. Efforts to identify the genetic basis of familial breast cancer reached fruition some years ago, when the breast-cancer susceptibility genes, BRCA1 and BRCA2 were identified. However, recent studies have suggested that mutations in these genes are associated with a smaller number (20 to 60%) of hereditary breast cancer families than originally estimated, especially in studies that have been based on population-based family materials. Several groups including ours are searching for additional breast cancer susceptibility genes using whole genome scanning approaches, but the success of many of these approaches depend on the underlying heterogeneity of the remaining cancer susceptibility loci. The failure to date to identify additional breast cancer susceptibility genes associated with a high risk of disease suggests that more than one may exist. We have taken the approach that the next BRCA genes will be those that encode for proteins whose functions are linked to important cell regulatory pathways. We have recently found one such candidate BRCA3 protein, referred to as p84N5.				
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## INTRODUCTION:

A major challenge to breast cancer researchers has been and continues to be the ability to distinguish genetic alterations that are critical to tumor initiation from those that are ephiphenomena of genetic instability. A certain number of breast cancer cases (~10%) are attributed to inherited mutations in highly penetrant breast cancer susceptibility genes, such as *BRCA1* and *BRCA2* [reviewed in (1)]. However, the majority of the tumors occur in women with little or no family history and the molecular basis of these sporadic breast cancers is still poorly defined. Amplification or over-expression of oncogenes (for example *c-MYC*, *ERBB2*, cyclin D1, *EGFR*,  $\gamma$ -synuclein) and loss of *TP53*, *PTEN* (phosphatase and tensin homolog deleted on chromosome 10), *PTCH* (patch), *MKK4* (MAP kinase kinase 4), *BRCA1*, *BRCA2*, and *HIN-1* (high in normal 1) have been shown to be present in sporadic disease (1-14). Epigenetic changes such as inactivation of *BRCA1* due to promoter hypermethylation have also been described in portion of breast carcinomas (15-17). We have recently discovered that the protein, referred to as p84N5 is abnormally expressed in the vast majority of breast cancer.

### 1.c Nuclear Matrix Protein, p84N5

The p84N5 gene, located on chromosome 18p11.32, was originally isolated on the basis of its ability to encode a protein that specifically associates with the N-terminal half of p110<sup>RB</sup> (18). This study demonstrated that p84N5 is a nuclear matrix protein that localizes to subnuclear regions associated with RNA processing and binds preferentially to the functionally active, hypophosphorylated form of p110<sup>RB</sup>. The relevance of this interaction for RB function is not completely understood. The p84N5 protein has a region of structural similarity to the death domains of several well-characterized proteins involved in apoptosis, including tumor necrosis factor receptor 1 (TNFR-1) (19). It is thought that p84N5 functions in an apoptotic-signaling pathway initiated from within the nucleus in response to DNA damage (19-21). In addition, the p84N5 protein in cell lines has a specific subcellular nuclear localization that gives a characteristic punctate staining pattern in cells (18). Furthermore, we have found that p84N5 does not appear to be expressed in normal breast ductal epithelial cells, but is expressed in the majority of breast tumor and tumor cell lines. A survey of the various hereditary cancer syndromes find that at least four are proto-oncogenes (i.e., *RET*, *MET*, *c-KIT*, *CDK4*) (22, 23). In general, activated oncogenes in the germline are usually embryonic lethal, yet these proteins are not. However, a second hit is observed (such as LOH) in the cancer, which leads to two mutant copies and/or trisomy (two mutant and one wild-type allele). We hypothesize that p84N5 may be a proto-oncogene, and when over-expressed or mutated contributes to the development of both sporadic and familial forms of breast cancer.

## BODY

### Progress report year 1

**Task 1 (Months 1-18).** To evaluate the expression of p84N5 in clinical breast tumor samples and correlation with predictive factors and clinical outcomes.

We have made substantial progress in terms of the first task. A paper has been prepared and will be submitted which has shown that p84N5 contributes human breast cancer progression and may be a predictive marker of disease progression and potential therapeutic target for treatment of the disease. Selected data from this paper is included below.

**Task 2 (Months 1-36).** To evaluate *BRCA1* and *BRCA2* mutation negative breast cancer-prone kindreds for germline p84N5 mutations.

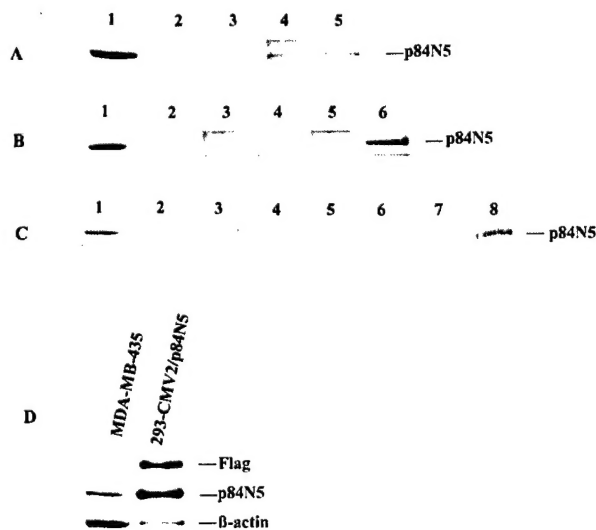
We have made limited progress in regards to this task and have screened only a small number of breast cancer cell lines for mutations in p84N5.

**Task 1-Progress Report**—"Evaluate the expression of p84N5 in clinical breast tumor samples and correlation with predictive factors and clinical outcomes."

### Characterization of Anti-p84N5 Antibodies

Two affinity-purified polyclonal rabbit antibodies (Ab), anti-p84N5 -N and -C were raised against p84N5 proteins encoding its N-terminal and C-terminal amino acids, respectively. To determine whether p84N5 antibody could immunoprecipitate intact p84N5, extracts of cells were immunoprecipitated with a control anti-IgG Ab or anti-p84N5-N, or -C. Blotted immunoprecipitates were probed with a commercial mouse monoclonal antibody (mAb) against p84N5, which recognizes N-terminal amino acids 15-373. The 84-kDa protein was detected in immunoprecipitated cell extracts by anti-p84N5 antibodies, thereby showing that the p84N5 antiserum can immunoprecipitate human p84N5 (**Figure 1a**). The 84-kDa protein was also detected in immunoprecipitated cell extracts by p84N5 mAb (**Figure 1b**) and Rb1 mAb (**Figure 1c**), but not BRCA2 mAb (data not shown). Moreover, the 84kDa protein was detected in a very high level in 293 cells which were transfected with CMV2/p84N5 plasmids (**Figure 1d**). These results demonstrate that the high specificity of p84N5 mAb.

Figure 1

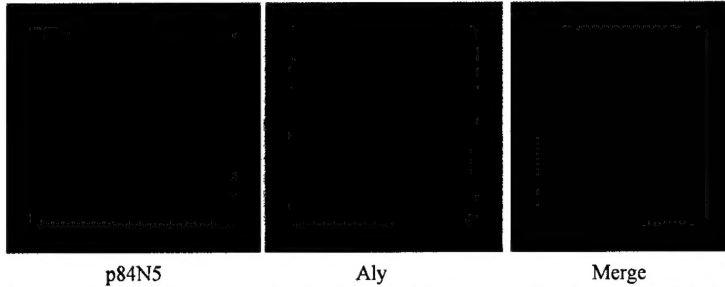


**Figure 1.** Validation of p84 antibodies. Protein samples were separated on an SDS-polyacrylamide gel and proteins were immunoblotted using anti-p84N5 monoclonal antibodies. (a) Lane 1, 50 µg of MDA-MB-435 cell lysates; Lanes 3, 4, 5, lysates immunoprecipitated with control normal rabbit IgG, C-terminal polyclonal anti-p84N5, and N-terminal polyclonal anti-p84N5 antibodies; Lane 2, blank. 500 µg of MDA-MB-435 cell lysates were used for the immunoprecipitations. (b) Lane 1, 50 µg of MDA-MB-231 cell lysates; Lane 2, 50 µg of normal breast tissue lysates; Lanes 3, 4, 500 µg of normal breast tissue lysates immunoprecipitated with control normal mouse IgG and anti-p84N5 mAb; respectively; Lanes 5, 6, 500 µg of MDA-MB-231 lysates immunoprecipitated with control normal mouse IgG, anti-p84N5 mAb, respectively. (c) Lane 1, 50 µg of MDA-MB-231 cell lysates; Lane 2, 50 µg of normal breast tissue lysates; Lane 3, blank; Lane 4, 5, 6, 500 µg of normal breast tissue lysates immunoprecipitated with control normal mouse IgG, anti-Rb1 mAb, and anti-p84N5 mAb, respectively; Lane 7, 8, 500 µg of MDA-MB-231 lysates immunoprecipitated with control normal mouse IgG, anti-Rb1 mAb, respectively. (d) Lane 1, 50 µg of MDA-MB-435 cell lysate; Lane 2, 15 µg cell lysate from 293 transfected with CMV2/p84N5 plasmid.

### Localization of p84N5 and co-localization with ALY

The cellular localization of p84N5 as well as co-localization with RB1 was demonstrated in CV-1 cells previously (Durfee et al., 1994). We confirmed whether p84N5 is located in nucleus and determined whether it co-localized with ALY in breast tumor cells. MDA-MB-231 cells were fixed, reacted with the p84N5 mAb and subsequently visualized via a fluorescently-labeled secondary antibody. As expected, p84N5 was detected exclusively in the nucleus of MDA-MB-231 cells (**Figure 2**). p84N5 was organized into multiple discrete foci and was not detected in the nucleolus. Two-color immunostaining was performed using anti-p84N5 and anti-ALY Abs and visualized by confocal microscopy. ALY

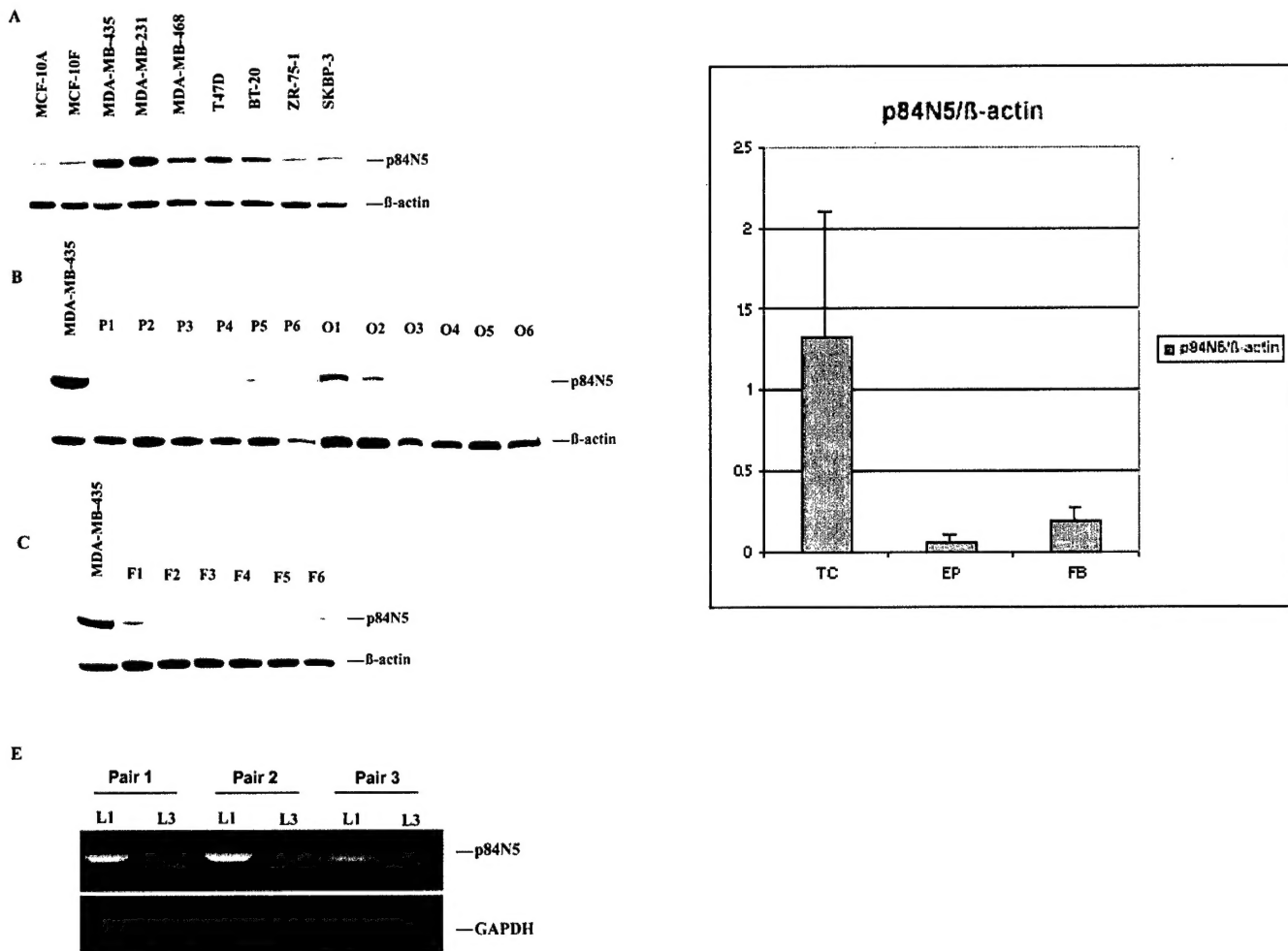
immunostaining revealed a nuclear dot pattern, consistent with previous work suggesting that ALY is a nuclear protein and co-localized with p84N5 (**Figure 2**).



**Figure 2.** Immunofluorescent visualization of p84N5 nuclear foci in MDA-MB-231 cells labeled with FITC. ALY immunostaining labeled with Texas and merged pictures showed co-localization with p84N5.

### **Expression of p84N5 is associated with human breast cell proliferation**

Previous studies of p84N5 have shown that forced p84N5 expression results in cell apoptosis. However, these studies lacked information regarding to the expression of endogenous p84N5 in human normal and tumor tissues. As a first step, we evaluated the expression of p84N5 in several breast tumor cell lines. We developed PCR primers that could amplify reverse transcribed mRNA specific to p84N5. Our initial screen of breast tumor cell lines found that all the cell lines expressed p84N5 mRNA (data not shown). We next evaluated these cell lines for expression of p84N5 by western blot analysis (**Figure 3a**). All the tumor cell lines expressed an 84-kDa protein. In contrast, 6 of 18 (33.3%) organoids, 6 primary epithelial cell cultures and 6 primary fibroblast cell cultures weakly expressed p84 (**Figure 3b & c**). We determined p84N5 expression (p84/ $\beta$ -actin ratio) quantitatively using the program IMAGE for each sample and found the p84N5 protein expression level (p84/ $\beta$ -actin ratio) in human breast cancer cell lines was significantly higher than those from primary epithelial and fibroblast cell cultures ( $p=0.0026$  &  $0.0044$ ) (**Figure 3d**). To test whether p84N5 expression may be associated with normal breast tissue development, we determined and compared p84N5 mRNA expression in 6 representative reduction mammoplasty specimens including 3 nulliparous premenopausal and 3 parous premenopausal women, by using a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). We found that p84N5 mRNA expression was much higher in the nulliparous specimens (**Figure 3e**).



**Figure 3.** Western blot and RT-PCR analysis of p84 in breast tissue samples. a-c) Protein samples were separated on an SDS-polyacrylamide gel and proteins were immunoblotted using anti-p84N5 or β-actin monoclonal antibodies. (a) p84N5 protein expression in several breast immortal cell lines, cancer cell lines, paired normal and breast cancer tissues. (b) p84N5 protein expression in primary breast epithelial cell cultures (P1~6) and organoids (O1~6). (c) p84N5 protein expression in primary breast fibroblast cell cultures. (d) p84N5/β-actin ratio in breast cancer cell lines (TC), primary breast epithelial cell cultures (EP), fibroblast cell cultures (FB). (e) Semi-quantitative RT-PCR. p84N5 mRNA expression in Lob 1 (L1) shows a more intense band than that in Lob3 (L3).

### Aberrant Expression of p84N5 is Associated with an Aggressive Phenotype of Human Breast Tumors

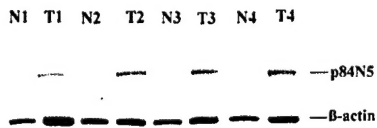
We evaluated a series of invasive breast tumors. The vast majority (71/72; 98.6%) of the tumors examined expressed p84 (**Figure 4** and data not shown). In comparison, only 8.1% (3/37) of normal adjacent breast tissues weakly expressed p84. The difference in p84 expression between normal and tumor tissues is significant ( $X^2=87.7$ ,  $p<0.001$ ). This observation suggests that p84N5 is not typically expressed in normal ductal epithelium but is present in the majority of invasive ductal carcinomas.

To further evaluate the potential role of p84N5 in breast tumor progression, we determined quantitatively p84N5 expression (p84/β-actin ratio) using the program IMAGE for each breast tumor sample. We found that p84N5 expression has a strong relationship with tumor size ( $p=0.015$ ), lymph node metastasis ( $p=0.002$ ), histologic grade ( $p=0.033$ ) as well as progesterone receptor status ( $p=0.011$ ), but not with menopausal status ( $p=0.375$ ) or estrogen receptor status ( $p=0.063$ ) (**Table 1**). These data indicate that p84N5 is associated with an aggressive phenotype of human breast tumors.



**Table 1.** Relationship between p84 expression and clinicopathologic parameters

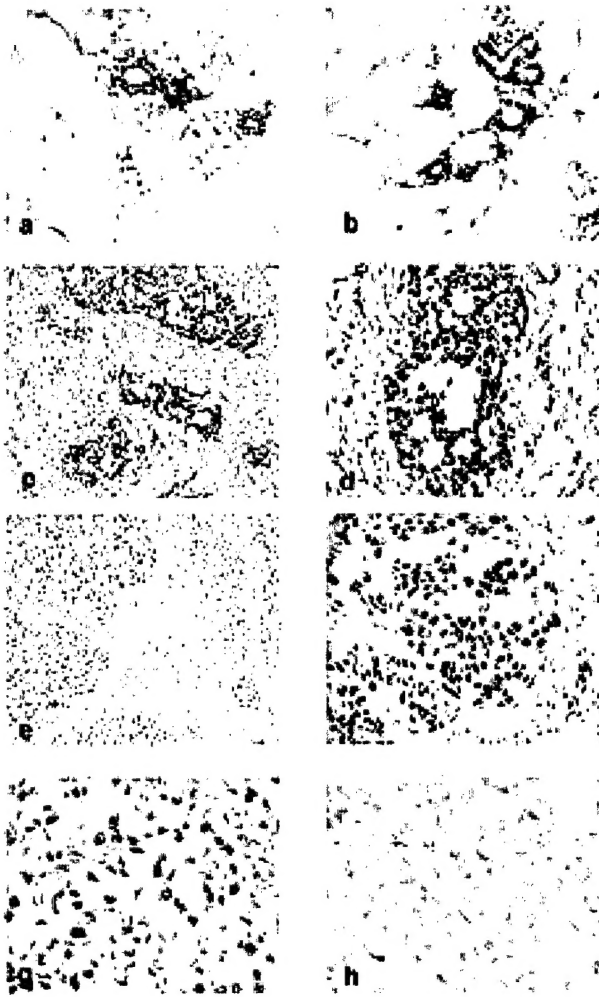
	n	Mean value	lb	ub	p value
Menopausal status					
Premenopausal	27	0.280	0.165	0.396	0.375
Postmenopausal	45	0.180	0.139	0.260	
Tumor size (cm)					
≤2	21	0.134	0.078	0.191	0.015
>2	50	0.285	0.234	0.363	
Lymph node metastasis					
Negative	31	0.131	0.077	0.185	0.002
Positive	36	0.329	0.232	0.425	
Histologic grade					
II	18	0.143	0.053	0.233	0.033
III	51	0.283	0.213	0.354	
Estrogen receptor					
Negative	23	0.32	0.193	0.448	0.063
Positive	36	0.177	0.122	0.247	
Progesterone receptor					
Negative	28	0.331	0.219	0.442	0.011
Positive	31	0.147	0.092	0.219	

**Figure 4.** p84N5 protein expression in paired normal and breast cancer tissues. Protein samples were separated on an SDS-polyacrylamide gel and proteins were immunoblotted using anti-p84N5 or  $\beta$ -actin monoclonal antibodies.

### p84N5 Immunoreactivity in Histologic Specimens

To further confirm that p84N5 is over-expressed in human breast cancer, we first utilized a commercial mouse monoclonal antibody (mAb) directed against p84N5 to detect its immunoreactivity in 10 cases of formalin-fixed, paraffin-embedded normal breast tissues which showed a high quality of reaction with PCNA and Ki67 in a previous report (Guo, et al., 2003), as well as in 50 cases of formalin-fixed, paraffin-embedded breast tumor samples. Only one positive case was found in breast tumors, and other samples failed to detect any positive signal. However, in freshly obtained and acetone-fixed tissues, p84N5 immunoreactivity was easily detected (**Figure 5**), although p84N5 immunoreactivity was much weaker (**Figure 5a**) in normal breast tissues than in invasive breast carcinomas (**Figure 5b**). As to the immunoreactive locations, p84N5 was strictly confined to the nuclei in low grade invasive breast carcinomas as expected, but in normal breast tissue, p84N5 was only weakly expressed in epithelial cells and in both cytoplasmic and nuclear distribution, myoepithelial cells were negative. Interestingly, p84N5 was also found in both cytoplasmic and nuclear distribution in some cases of high grade invasive breast carcinomas.



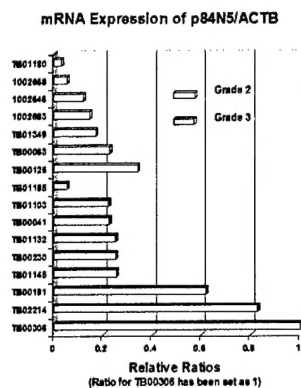


**Figure 5.** Immunohistochemical stains of normal breast tissue and breast tumor specimens for the p84N5 protein. Magnification: approximately 100 X (a, b, c, e); 200X (d, f, g, h). (a, b) p84N5 weakly expressed in the cytoplasm and nuclei of normal ductal epithelia and lobular epithelia. (c, d) p84N5 expressed in the cytoplasm and nuclei of a grade I invasive ductal carcinoma. (e, f) p84N5 highly expressed in the nuclei of a grade II invasive ductal carcinoma. (g) p84N5 expression exclusively in the nuclei of a grade III invasive ductal carcinoma. (h) a negative control, secondary antibody only.

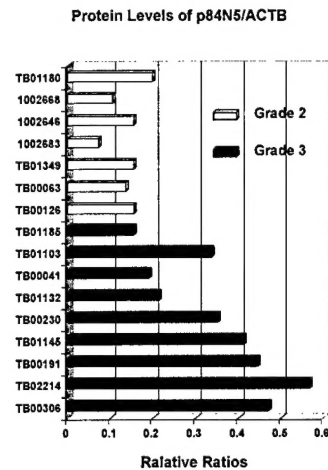
### Aberrant mRNA expression of p84N5 in breast tumors

Quantitative real-time PCR (qPCR) was also performed to evaluate the expression of p84N5 mRNA levels in subset of above tumor samples. As seen from **Figure 6a & b**, both mRNA and protein levels in tumors of grade III are relatively higher than those of grade II. To further validate the expression of p84N5 in breast tumors, we performed qPCR analysis on laser captured micro-dissection (LCM)-purified normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells (**Figure 6c & d**). Three normal breast tissues and 7 invasive ductal carcinomas were micro-dissected and the RNA evaluated by RT-PCR. We found that 85% of these tumors (6 of 7) showed elevated levels of *P84N5* mRNA relative to the normal mammary ductal epithelium. As shown from **Figure 6d**, late stage tumors expressed the highest levels of *p84N5* mRNA as compared to normal mammary ductal epithelium and stage I/II tumors.

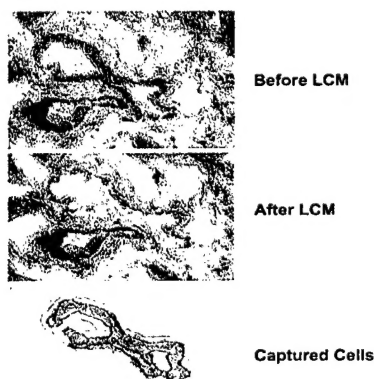
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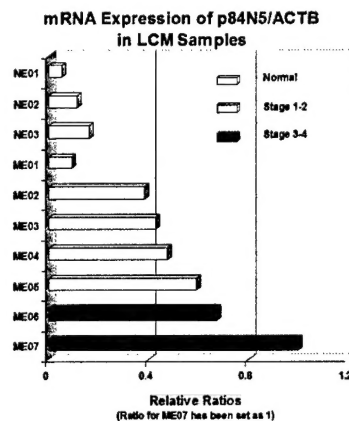
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C.



D.



**Figure 6.** Analysis of p84N5 expression in breast tumor samples. (A) mRNA expressions analyzed by quantitative real-time PCR; (B) protein levels analyzed by western blot. Both mRNA expression and protein levels were adjusted to  $\beta$ -actin levels. Analysis of p84N5 expression in laser capture microdissection (LCM) breast samples. (C) Depiction of mammary epithelial cells before and after laser capture micro-dissection. (D) Quantitative real-time PCR was used to analyze normal mammary ductal epithelial cells (NE) and malignant epithelial (ME) cells captured by micro-dissection.

### Small interfering RNAs directed against p84N5 inhibit *in vitro* proliferation in MDA-MB-231

Since p84N5 over-expression was associated with a more aggressive phenotype of breast cancer, we sought to determine if over-expression may have a role in regulating the proliferation of established breast cancer cells. Therefore we used RNAi to abrogate p84N5 expression in the MDA-MB-231 cell line and evaluate the cells for altered growth. Two sets of siRNA duplexes were designed for different coding regions, i.e., nucleotides 710-730 and 1652-1672. In addition, RNA oligonucleotides without homology to human or animal genes and with the same percentage of guanines and cytosines (G/C content) as the above two sets were used as a control. Each of these annealed RNAs was transfected into the MDA-MB-231 cells and their effects on p84N5 protein levels were compared to mock-transfected cells by Western blot analysis at 72 h posttransfection (**Figure 7**).

Both siRNAs effectively reduced the levels of p84N5, but did not apparently affect the levels of  $\beta$ -actin (**Figure 7a**). The p84N5 protein level was reduced by p84N5-siRNA(a) by 62.4%. p84N5-siRNA(b) was more effective, suppressing p84N5 protein levels by 87.2%. Immunostaining confirmed that p84N5 protein was drastically decreased in majority of the treated cells (**Figure 7b**). Consistent with

the protein level change, RNA levels were also reduced by using semi-quantitated RT-PCR (**Figure 7c**). These results indicate that siRNA can markedly reduce p84N5 level in cancer cell lines that have elevated levels of this protein.

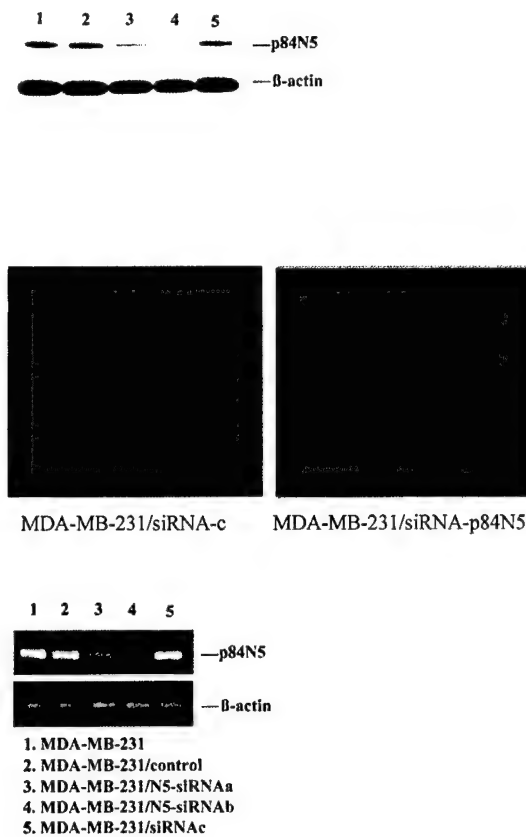
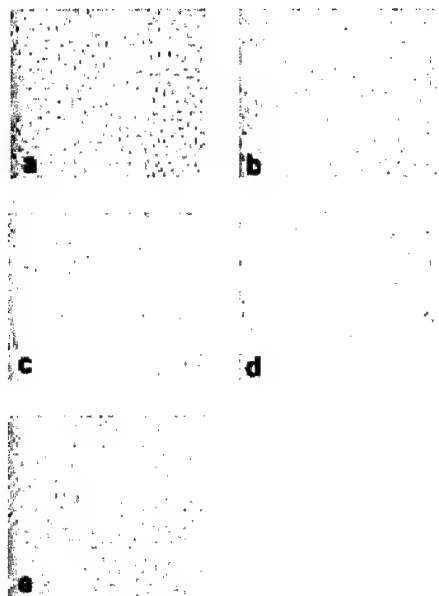


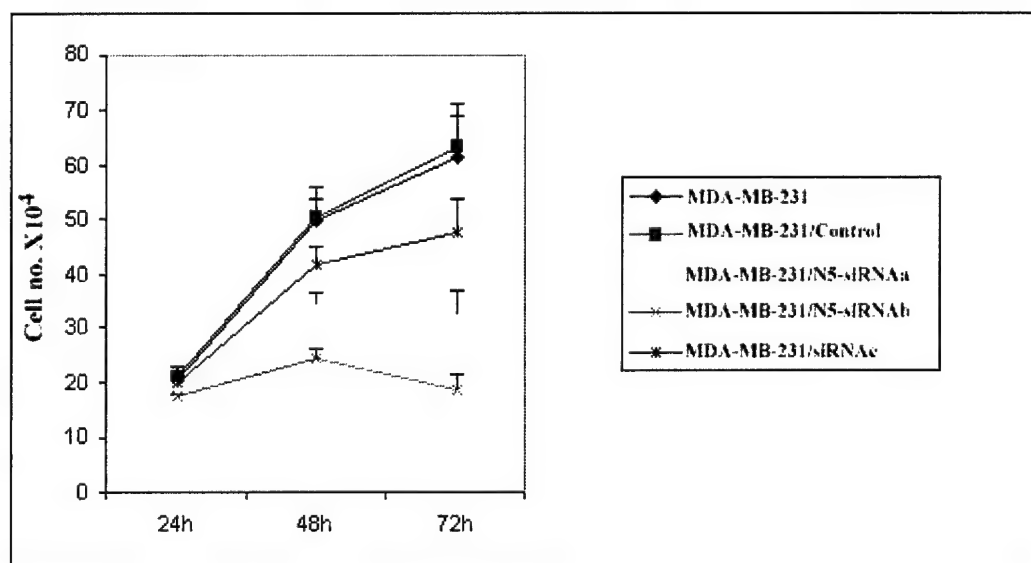
Figure 7. Western blot analysis. MDA-MB-231 protein samples were separated on an SDS-polyacrylamide gel and proteins were immunoblotted using anti-p84N5 or  $\beta$ -actin monoclonal antibodies. Lane 1, cells without any treatment; lane 2, cells treated with transfection reagent, Lane 3, 4, 5 treated with p84N5-siRNA(a), p84N5-siRNA(b) and control-siRNA, respectively. b. Immunostaining of p84N5 in MDA-MB-231 treated with control siRNA and p84N5-siRNA, respectively. c. Semi-quantitative RT-PCR detection of p84N5 mRNA expression in MDA-MB-231 cell lines treated and untreated with siRNA.

We next examined whether the siRNA-mediated decreases in p84N5 protein in the MDA-MB-231 cells are accompanied by a reduction of cell proliferation. Visually, the total numbers of the cells decreased significantly following treatment with p84N5-siRNAs as compared to cells treated with transfection reagent or control-siRNA (**Figure 8**). Guava ViaCount assays confirmed that there was a reduction in cells treated with p84N5-siRNAs as compared to cells treated with control siRNA or only the transfection reagent (**Figure 9**) and the differences are statistically significant ( $p < 0.01$ ). As is consistent with the protein reduction, p84N5-siRNA(b) was also more efficient in the cell number reduction than p84N5-siRNA(a). Probably due to nonsepecific cell toxicity by siRNA transfection, the cells treated with control siRNA also showed a slight reduction in viable cells. The viability of cells treated with p84N5-siRNAs was also decreased as compared to cells treated with control siRNA or mock transfected, however, the differences were not significant ( $p > 0.05$ ) (data not shown). GuavaNexin assay showed that there was also a reduction of Annexin V-PE and 7-AAD positive cells in cells treated with p84N5-siRNAs as compared to controls and the differences were also not significant ( $p > 0.05$ ) (data not shown). Similar results with p84N5 siRNAs were also seen in other tumor cell lines including OVCAR5 and OVCAR10 (data not shown). In order to determine the mechanism of p84N5 siRNA action, we further determined the cell cycle distribution by flow cytometry and found that the cell numbers in G2-M phase were decreased and cell numbers in G1 phase were increased in MDA-MB-231 treated with p84N5 siRNA comparing with the cells treated with control siRNA, indicating that p84N5 may be necessary for

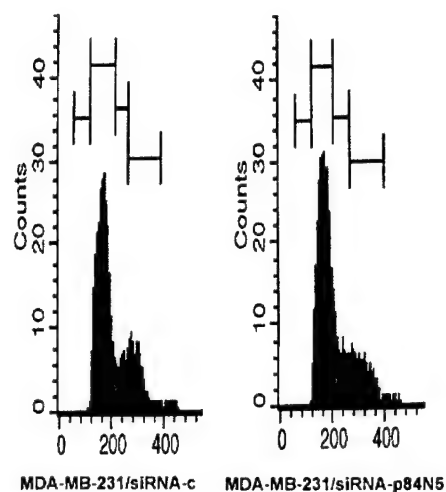
entry into the G2-M phase (**Figure 10**). Overall, these results strongly suggest that the inhibitory effect of p84N5-siRNA on endogenous p84N5 protein expression and its biologic consequences apparently lead to reduction in cell proliferation, probably due to p84N5-siRNA blocking entry into G2-M phase of the cell cycle.



**Figure 8.** The photomicrographs of morphologic appearance of the MDA-MB-231 cells. (a) cells without any treatment, (b) cells treated with transfection reagent only, (c, d) cells treated with p84N5-siRNA, (e) control-siRNA, respectively.



**Figure 9.** Viable cell numbers of the MDA-MB-231 cells treated with p84-siRNA or control siRNA (Figure 8).



**Figure 10.** FACS analysis of breast tumor cells following down-regulation of p84N5 levels. The comparison of MDA-MB-231 cell cycle distribution after 72 hour of treatment with either control siRNA (left panel) or p85N5-siRNA (right panel).

## **C- KEY RESEARCH ACCOMPLISHMENTS:**

### **C.1. “The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene”**

- 1.a. Demonstrated that p84N5 is expressed at very low levels in normal breast epithelial cells and that it is highly expressed in breast tumors.
- 1.b. Report that p84 expression correlates with tumor size and the metastatic state of the tumor progression, i.e., identify p84 as a prognostic marker for aggressive forms of human breast cancer.
- 1.c. Demonstrated that reduction of p84 levels in breast cancer cell lines by siRNA results in inhibition of cellular proliferation.

**D-REPORTABLE OUTCOMES (5/2003 to present):**

***D.1. "The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene"***

**1.a. Abstracts**

Shan-Chun Guo and A.K. Godwin. Accumulation of p84N5 domain protein is associated with an aggressive phenotype of human breast tumors. Proceedings of American Association of Cancer Research, 44:2421, 2003.

Guo, S., Farber, M.J., Shiekhattar, R. and Godwin, A.K. Over-expression of death domain containing protein-p84N5 in human ovarian cancer cell lines is associated with cell proliferation. Proceedings of American Association of Cancer Research, 44:1805, 2004.

**1.b. Publications**

Guo, S., Hakimi, M-A., Baillat, D., Chen, X., Farber, M.J., Klein-Szanto, A.J.P., Cooch, N.S., Shiekhattar, R., Godwin, A.K. Linking Transcriptional Elongation and mRNA Export to Metastatic Breast Cancers. In preparation, 2004.

**Book chapters and review articles:**

Pan, Z-Z., and Godwin, A.K. Oncogenes, Cancer, and Targeted Therapy. Life and Analytical Science, accepted, 2004.

## **E-CONCLUSIONS:**

### ***E.1. "The nuclear death domain protein p84N5; a candidate breast cancer susceptibility gene"***

The biochemical pathways that are disrupted in the genesis of sporadic breast cancers remain unclear. Moreover, the present prognosticating markers used to determine the prognosis of node-negative-patient leads to probabilistic results and the eventual clinical course is far from certain. Here we identified the human TREX complex, a multi-protein complex that links transcription elongation to messenger RNA transport, as the culprit for aggressive human breast cancers. We show that while p84 is expressed at very low levels in normal breast epithelial cells, it is highly expressed in breast tumors. Importantly, p84 expression correlates with tumor size and the metastatic state of the tumor progression. Reduction of p84 levels in breast cancer cell lines by siRNA result in inhibition of cellular proliferation. These results not only identify p84 as a prognosticator of breast cancer but also delineates human the TREX complex as a target for therapeutic drugs against breast cancer.



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G-APPENDIX - None